Contractile Activity Regulates Isoform Expression and Polysialylation of NCAM in Cultured Myotubes: Involvement of Ca^{2+} and Protein Kinase C

Victor F. Rafuse and Lynn Landmesser

Department of Neurosciences, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106-4975

Abstract. Muscle development involves a series of complex cell–cell interactions that are mediated, at least in part, by several different cell adhesion molecules. Previous work from this lab showed that the different isoforms of NCAM and its level of polysialylation are developmentally regulated during chick myogenesis in vivo and that this regulation is important for normal muscle development. Using developing chick secondary myotubes grown in culture, we show here that both the polysialylation of NCAM and the developmental switch in isoform expression are regulated by activity and that Ca^{2+} entry through voltage-gated channels and the subsequent activation of protein kinase C are required for the developmental changes in NCAM isoform synthesis. Specifically, PSA expression was shown to be developmentally regulated with high expression being temporally correlated with the onset of spontaneous contractile activity. Furthermore, blocking contractile activity caused a decrease in PSA expression, while increasing activity with electrical stimulation resulted in its up-regulation. Immunoblot and metabolic labeling studies indicated that dividing myoblasts synthesize primarily 145-kD NCAM, newly formed, spontaneously contracting myotubes synthesize 130-, 145-, and 155-kD NCAM isoforms, while older, more mature myotubes primarily synthesize the glycosylphosphatidylinositol-anchored 130-kD isoform. In addition, mature myotubes synthesized a 180-kD isoform which, in contrast to the other three isoforms, had a high rate of turnover. This developmental switch in NCAM isoform expression could be inhibited with Ca^{2+} channel blockers and inhibitors of protein kinase C. Taken together, these results suggest that Ca^{2+} ions and protein kinase C are involved in a second messenger cascade coupling membrane depolarization with transcriptional factors that regulate NCAM isoform synthesis and polysialylation.

THE neural cell adhesion molecule (NCAM)[1] is developmentally expressed on a wide variety of cell types and mediates many cell–cell interactions including axonal growth (Doherty and Walsh, 1992), fasciculation (Landmesser et al., 1988), cell migration (Ono et al., 1994), and myogenesis (reviewed by McDonald et al., 1995). During skeletal muscle development, NCAM is expressed at the onset of primary myogenesis, increases throughout secondary myogenesis and eventually declines around the time of birth (Covault and Sanes, 1986) or hatching (Fredette et al., 1993; Tosney et al., 1986). Skeletal muscle NCAM has at least three major isoforms that are generated by alternative splicing of a pre-mRNA that is transcribed from a single gene (Cunningham et al., 1987; Owens et al., 1987). Proliferating myoblasts in vitro (Covault et al., 1986; Moore et al., 1987; Knudsen et al., 1989) and primary myotubes in vivo (Fredette et al., 1993) predominantly express the 145-kD transmembrane isoform of NCAM. However, as development proceeds, both in vivo and in culture, the 145-kD isoform is down-regulated and the GPI-linked 130-kD isoform of NCAM is up-regulated to become the predominant isoform (Covault et al., 1986; Fredette et al., 1993; Yoshimi et al., 1993). In vivo, this large upregulation of the GPI-linked isoform is associated with secondary myogenesis (Fredette et al., 1993).

The factors that regulate the expression of NCAM, and in particular its different isoforms, are not well understood. NCAM on mammalian skeletal muscle fibers is down-regulated postnatally but is rapidly re-expressed following denervation (Covault and Sanes, 1985, 1986). These results indicate that the overall expression of NCAM in neonatal and adult muscle fibers is regulated by muscle activity. However, the role of neuromuscular activity in regulating the expression of individual isoforms during em-

Address correspondence to Dr. Lynn Landmesser, Dept. of Neurosciences, Case Western Reserve University, Cleveland, OH 44106-4975. Tel.: (216) 368-8448. Fax: (216) 368-4650.

1. *Abbreviations used in this paper*: AChR, acetylcholine receptors; dTC, d-tubocurarine; MSD, muscle-specific domain; NCAM, neural cell adhesion molecule; PKC, protein kinase C; PNA, peanut agglutinin; PSA, polysialic acid; TXX, tetrodotoxin; SR, sarcoplasmic reticulum.

bryogenesis is less clear. Blockade of neuromuscular activity during primary and secondary myogenesis in vivo prevents the normal switch from the 145- to the 130-kD isoform of NCAM (Fredette et al., 1993), suggesting that activity plays a role in regulating this isoform switch. However, since activity blockade also results in a dramatic reduction in the total number of secondary myotubes that are formed (Fredette and Landmesser, 1991; Harris, 1981; Ross et al., 1987), the observed absence of an isoform switch in activity blocked muscles may simply result because secondary myogenesis is inhibited. Thus it is not possible to conclude from these observations that activity directly affects NCAM isoform expression.

Electrical activation of myotubes has also been shown to regulate the polysialylation of NCAM. During early stages of in vivo myogenesis polysialic acid (PSA) on NCAM is low, but as myogenesis proceeds and neuromuscular activity increases, PSA is dramatically up-regulated on myotubes and then declines (Fredette et al., 1993). Blockade of spontaneous neuromuscular activity by chronic treatment with d-tubocurarine (dTC) prevented both the expression of PSA on myogenic cells and the separation of myotubes from clusters (Fredette et al., 1993).

How might electrical activity regulate NCAM isoform expression and PSA? Neuromuscular activity is known to be an important factor regulating the expression of acetylcholine receptors (AChRs) both in vivo (reviewed by Changeux et al., 1990) and in vitro (Betz and Changeux, 1979; Changeux et al., 1990). More recent studies have shown that the activity dependent down regulation of AChRs is blocked by both voltage-dependent Ca^{2+} channel blockers (Fontaine et al., 1987; Klarsfeld et al., 1989; Huang, et al., 1994) and by inhibitors of protein kinase C (PKC) (Klarsfeld et al., 1989; Huang et al., 1992). Since the expression of both AChRs and total NCAM is down-regulated by neuromuscular activity, and up-regulated in the absence of activity, it is possible that both proteins share a common regulatory pathway.

To examine more closely the role of neuromuscular activity and potential downstream molecular mechanisms regulating the expression of both NCAM isoforms and of PSA, chick secondary myotubes grown in culture were subjected to a number of controlled experimental paradigms designed to alter activity and/or downstream second messengers. We find that the polysialylation of NCAM and the developmental switch in isoform expression is regulated by activity. Furthermore, Ca^{2+} entry through voltaged gated channels and the activation of PKC is required for the developmental changes in NCAM isoform synthesis.

Preliminary results have previously been published in abstract form (Rafuse, V., and L. Landmesser. 1994. *Soc. Neurosci. Abstr.* 20:1318).

Materials and Methods

Cell Culture

Myotube cultures were isolated from white leg horn chick pectoral muscles from stage 38 (Hamburger and Hamilton, 1951) embryos. Cultures prepared for immunofluorescence and metabolic labeling were prepared with the following procedure. Myoblasts were mechanically dissociated from myotubes by gently triturating the dissected muscles through two fire-polished Pasteur pipettes with openings of 0.75 and 0.5 mm. The cell suspension was centrifuged at 1,000 *g* for 15 s to separate the myoblasts from cell debris. The supernatant containing the myoblasts was diluted with culture medium to a final concentration of 10^6 myoblasts/ml. Myoblasts were plated on rat tail collagen (type I; Collaborative Biomedical Products, Bedford, MA)-coated glass cover slips (12 mm; Carolina Biological Supply Company) at 50,000 cells/cover slip in 0.5 ml of Ham's F-10 (supplemented with 1.26 mM $CaCl_2$) containing 10% horse serum (GIBCO BRL, Gaithersburg, MD), 5% chicken embryo extract, 100 U/ml penicillin, and 100 μg/ml streptomycin (culture medium) and cultured in 24-well plastic tissue culture plates. 50% of the culture medium was replaced with fresh medium on day 3 and thereafter every 2 d. Under these conditions myotubes routinely began spontaneously contracting on day 3.

Cultures used for SDS-PAGE immunoblot analysis were prepared as above except that myoblasts were plated in collagen-coated 60 mm plastic culture dishes at 10^6 cells/dish in 5 ml medium. No differences were observed in either the morphology or rate of myogenesis between the two culture conditions. As observed with the cultures plated on glass coverslips, myotubes began spontaneously contracting on day 3.

To examine the effects of electrical activity on NCAM and PSA expression, cultured myotubes were either treated with tetrodotoxin (TTX) or electrically stimulated. Spontaneous contractile activity was blocked by treatment with 5 μM TTX (added on day 2) or was increased by electrically stimulating 7 d cultured myotubes. As described above, myotubes were cultured in 24-well tissue culture plates on collagen-coated glass coverslips. Stimulating silver electrodes were immersed in the culture medium and placed on opposites sides of a single well containing spontaneously contracting myotubes. Myotubes were stimulated using a stimulator (model S88; Grass Instruments Co., Quincy, MA) that was coupled to an integrated circuit (IC) output device that converted monopolar pulses to balanced bipolar pulses. The output from the IC device was connected to the silver stimulating electrodes with flexible copper wires. The myotubes were stimulated with a single pulse (1-ms pulse width at 60 V) every 10 s for 3 d. This procedure effectively stimulated ~60% of the cultured myotubes. Myotubes that did not contract when electrically stimulated were also never seen to contract spontaneously. The electrical stimulation parameters used did not have any detrimental effects on the health of the myotubes based on both their morphology and frequency of spontaneous contractions.

Immunofluorescence

Three antibodies to NCAM were used: 5E, a monoclonal IgG antibody that recognizes all isoforms of NCAM (Frelinger and Rutishauser, 1986), 4D, a monoclonal IgG antibody that only recognizes the 180-kD NCAM isoform (Watanabe et al., 1986), and 5A5, a monoclonal IgM that recognizes only the polysialated form of NCAM (Dodd et al., 1988; Acheson et al., 1991). For double labeling of both total and PSA forms of NCAM, 5E and 5A5 were diluted together in PBS (pH 8.0) and BSA to a final concentration of 1:100 each.

Cultured cells were washed with PBS and then fixed with cold 3.7% formaldehyde for 15 min at room temperature. Fixed cells were washed six times (5 min/wash) with PBS and then blocked with 2% BSA/PBS for 1 h at room temperature. All cells were processed for immunohistochemistry by first incubating them overnight at 4°C in primary antibodies diluted in 2% BSA/PBS. After rinsing with PBS the cells were incubated for 1.5 h in a mixture of secondary antibodies (Sigma Immunochemicals, St. Louis, MO) consisting of fluorescence-conjugated anti-mouse IgG (1:100) and rhodamine-conjugated anti-mouse IgM (1:100). After rinsing several times in PBS the coverslips were mounted on a slide and coverslipped with a glycerol and PBS mixture (50:50) containing *p*-phenylenediamine (0.3 mg/ml) to prevent fading. All comparisons for staining intensity were performed using the same dilution of antibodies and photographed with Kodak Tri-X Pan film using the same exposure settings. Photographs were taken with a Nikon Microphot microscope equipped with epifluorescence.

SDS-PAGE Immunoblotting

Cultured cells were washed with PBS, harvested in 4 ml of PBS and centrifuged at 5,000 *g* for 10 min at 4°C. The pellet was re-suspended in extraction buffer (50 mM Hepes, 150 mM NaCl, 1mM EDTA, 2 mM PMSF, 100 μg/ml leupeptin, 0.2 TIU/ml aprotinin, 1% NP-40) sonicated for 10 s, placed on ice for 1 h and centrifuged for 1 h at 100,000 *g* (4°C). The concentration of the solubilized proteins was determined (BCA method; Pierce Chemical Co., Rockford, IL) and adjusted to 1 mg/ml. Some aliquots of the extract (25 μl) were treated with neuraminidase (0.2 U/ml of

Vibrio Cholerae; Calbiochem-Behring Corp., San Diego, CA) for 2–3 h at 37°C to remove sialic acid while others from the same sample were treated with buffer alone. SDS sample buffer containing dithiothreitol was added to each sample, heated for 20 min at 65°C, and then the proteins were separated by SDS-PAGE according to the Laemmli method (Laemmli, 1970) on a 6% gel. 20 μg of total protein was loaded in each lane. Proteins were transferred onto Immobilon-P membranes, incubated for 1 h with blocking solution (4% milk in TBS), followed by overnight incubation at 4°C with 5E diluted in TBS and 1% milk (1:500). Membranes were washed several times with TBS and then they were incubated for 1.5 h with alkaline phosphatase-conjugated mouse IgG secondary antibody (1:500; Sigma Immunochemicals). Bands were visualized with the BCIP/NBT method.

Peanut Lectin Immunoblots

5-d-old myotube cultures were washed with PBS, harvested and homogenized in 200 μl of extraction buffer as described above. The extracted proteins were rotated overnight at 4°C in the presence of 30 μl of 5E-conjugated Sepharose beads to immunoprecipitate NCAM. Beads were washed four times (1 ml each) with buffer A (50 mM Tris-HCl, 0.5 M NaCl, 1% NP-40, pH 8), four times with buffer B (50 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 0.1% SDS, pH 8) and one time with extraction buffer (see above). Beads were diluted with 30 μl of extraction buffer and half the sample was incubated with neuraminidase (see above) for 1–2 h at 37°C to remove the sialic acid. SDS sample buffer containing dithiothreitol was added to each sample, heated for 20 min at 65°C, and then the proteins were separated by SDS-PAGE on a 6% gel. Proteins were transferred onto Immobilon-P membranes and were incubated for 1 h with blocking solution (4% milk in TBS). The membranes were then incubated overnight at room temperature with peanut agglutinin (PNA) (10 μg/ml; Sigma Immunochemicals). PNA was diluted in Hepes-buffered saline (10 mM Hepes, 0.15 M NaCl, 0.1 mM $CaCl_2$) containing 1% BSA. Treated membranes were then washed several times with TBS, incubated for 1 h with anti-PNA (anti-arachis hypogaea lectin) (1:10; Sigma Immunochemicals), washed, and finally incubated for 1.5 h with alkaline phosphatase-conjugated rabbit IgG secondary antibody (1:500; Sigma Immunochemicals). Bands were visualized with the BCIP/NBT method. Each lane on the immunoblot represents half of the total NCAM immunoprecipitated from myotubes cultured in a single 60 mm tissue culture dish.

Metabolic Labeling

Glass coverslips, containing myotube cultures, were placed in 24-well tissue culture plates containing 250 μl of methionine-free MEM (Sigma Immunochemicals) for 1 h at 37°C prior to addition of 30 μCi of [^{35}S]methionine. Cultures were pulsed for 8 h at 37°C after which the cells were washed at least 15 times (2 ml each) with cold MEM. Washed cells were either homogenized immediately or were placed in fresh culture medium (see above) and then incubated (chased) for 1 or 2 d. Cells to be homogenized were scraped from the coverslip, added to 200 μl extraction buffer (see above), sonicated for 10 s, placed on ice for 1 h, and finally centrifuged at 100,000 *g* for 1 h at 4°C. Vials containing extracted proteins and 30 μl of 5E-conjugated Sepharose beads were rotated overnight at 4°C to immunoprecipitate NCAM. Beads were washed four times (1 ml each) with buffer A, four times with buffer B, and once with extraction buffer (see above). Beads were diluted with 30 μl of extraction buffer and incubated with neuraminidase (see above) for 1–2 h at 37°C. SDS sample buffer was added to each sample, heated for 20 min at 65°C, and then the proteins were separated by SDS-PAGE on a 6% gel. Equivalent amounts of protein were added to each lane, which represents half of the total NCAM immunoprecipitated from myotubes cultured on a single 12 mm glass cover slip. Gels were fixed (10% acetic acid, 25% isopropanol, 65% water) for 30 min, soaked in Amplify (Amersham Corp., Arlington Heights, IL) for 30 min, and dried on blotting paper using a Bio-Rad drier (gel drier model 583; Bio-Rad Laboratories). Dried gels were placed onto pre-flashed Hyperfilm-MP (Amersham Corp.) in an X-ray cassette for 1 wk at −70°C after which the films were developed using a medical film processor (model QX-70; Konica).

Drug Treatments

Several pharmaceutical agents were used throughout this study to alter normal cellular function. Voltage-sensitive calcium channels were blocked with 10 μM verapamil (Calbiochem-Behring Corp.) (Walsh et al., 1986). To increase intracellular Ca^{2+} levels myotubes were treated with either 10 mM thapsigargin (Calbiochem-Behring Corp.) an inhibitor of Ca^{2+}-ATPase activity (Thastrup et al., 1990) or with veratradine, a Na channel activator (Stallcup, 1977). PKC was inhibited with either 10^{-6} M phorbol 12 myristate-13-acetate (TPA; Sigma Immunochemicals) (Tamaoki et al., 1986) or 10^{-7} M staurosporine. Finally, phosphatidycholine-specific phospholipase C was selectively inhibited with 30 mM D609 (Calbiochem-Behring Corp.) (Muller-Decker, 1989). Individual drugs were added to the myotube cultures on day 4 and remained in the medium for the described times.

Results

Immunocytochemical Comparison of Total NCAM and Polysialic Acid Expression during Secondary Myogenesis

Previous work in this laboratory (Fredette et al., 1993) showed that, while total NCAM is relatively uniformly expressed on developing chick myoblasts and myotubes throughout primary and early secondary myogenesis in vivo (St 28-40), the amount of PSA associated with NCAM was temporally regulated. Specifically, the intensity of PSA immunofluorescence was very low during early primary myogenesis (St 28), increased slowly during late primary and early secondary myogenesis, until the beginning of St 37, at which time PSA immunostaining sharply peaked (St 38) correlating with the break up of myotube clusters. To better clarify the mechanisms regulating the temporal pattern of NCAM and PSA expression on developing chick muscle in vivo, we used an in vitro model of secondary myogenesis where putative regulatory factors could be more easily controlled.

The formation of myotubes in vitro occurs in a series of well-timed events. During the first 24 h of myogenesis, myoblasts divided and many began to align into small clusters of cells. One day after plating immunofluorescence for total NCAM was uniformily distributed along the surfaces of all myoblasts (Fig. 1 *A*). During the next 24 h of myogenesis myoblasts continued to divide and tightly align as long strings of cells that began to take the shape of the future myotubes. The expression of total NCAM remained high during this time period with slightly higher immunostaining observed between apposed cells (Fig. 1 *C*). Over the next 24 h virtually all myoblasts fused together to form large multinucleated myotubes. When myoblasts were cultured in Ca^{2+} supplemented F-10 medium, as was done in this study, many newly formed myotubes began to exhibit frequent spontaneous contractions by the third day of culture and many, but not all, remained spontaneously active for as long as the myotubes remained in culture. During this entire period, overall NCAM staining remained high on both newly formed, as well as on older, more mature myotubes (Fig. 1, *E* and *G*). Thus, in vitro, overall NCAM immunostaining did not appear to be developmentally regulated or to be affected by the onset of spontaneous contractility in myotubes.

In contrast, PSA expression was strongly correlated with the onset on electrical activity. Thus, PSA was absent from myoblasts at 1 d after plating (Fig. 1 *B*) and was only weakly expressed on a few myoblasts at 2 d (Fig. 1 *D*). At 3–4 d, when myotubes became spontaneously active, PSA was expressed at moderate levels by many, but not all (Fig. 1 *F*) myotubes. During the next few days PSA levels increased with some myotubes exhibiting intense staining

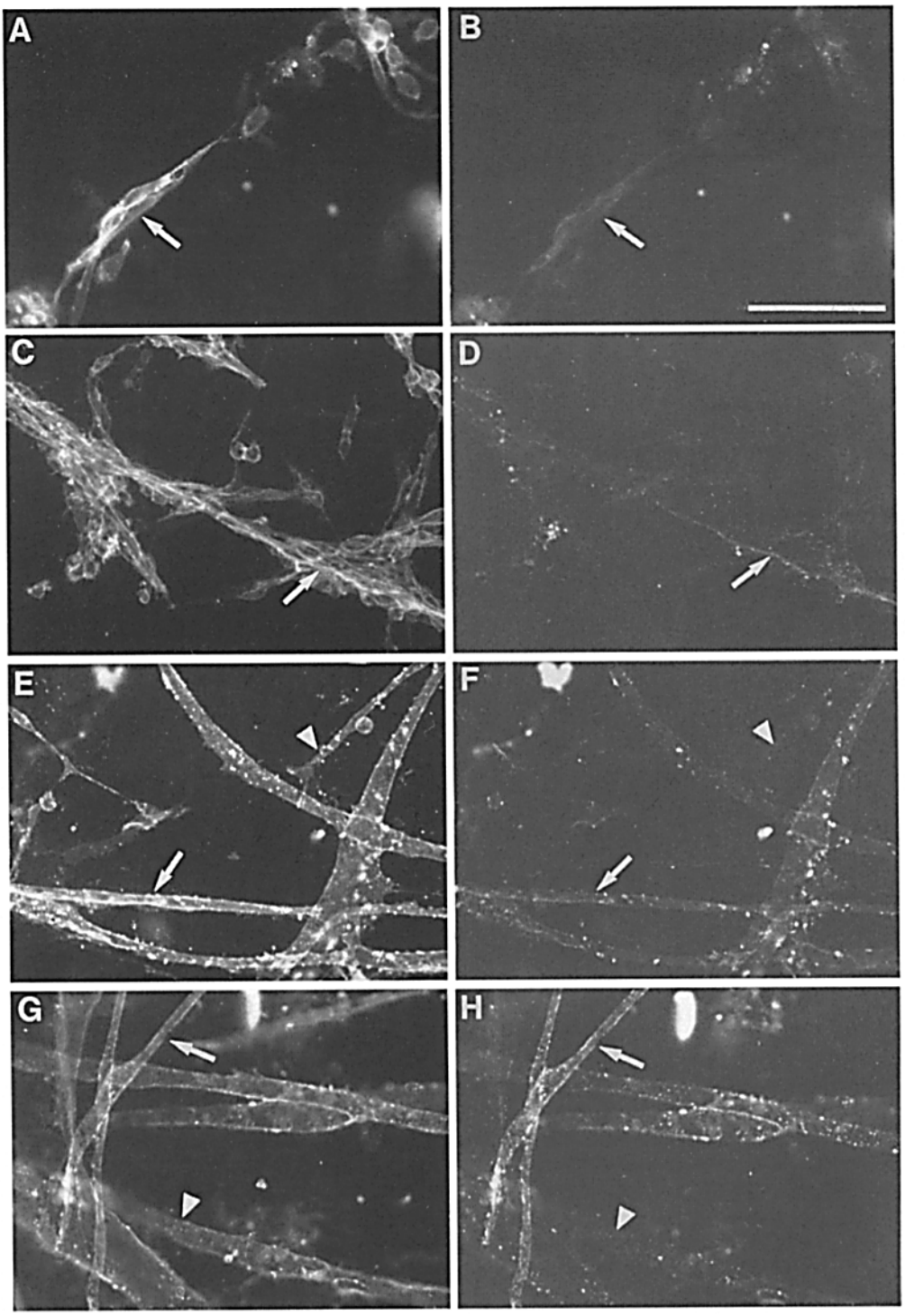

Figure 1. NCAM and PSA expression during different stages of in vitro myogenesis. Cultures were immunostained for NCAM (*A*, *C*, *E*, and *G*) or PSA (*B*, *D*, *F*, and *H*). At 1 d after plating myoblasts express NCAM strongly (*A*, *arrow*), but PSA is weak to absent (*B*, *arrow*). At 2 d NCAM is strongly expressed on all myoblasts (*C*, *arrow*) and PSA begins to be weakly expressed on a few fusing myoblasts (*D*, *arrow*). By 3–4 d NCAM staining is intense on the newly formed myotubes (*E*, *arrow*). PSA expression increases on most myotubes (*F*, *arrow*), but is weak to absent on others (*F*, *arrowhead*) which are NCAM positive (*E*, *arrowhead*). By 8 d PSA expression has increased markedly on many myotubes (*H*, *arrow*), but is still low to absent on others (*H*, *arrowhead*). In contrast, NCAM staining is uniformly high on all myotubes (*G*, *arrow* and *arrowhead*). Bar, 75 μm.

(Fig. 1 *H*, *arrow*). However, PSA staining remained heterogeneous and even by day 8 some myotubes which expressed NCAM still exhibited weak to absent staining for PSA (Fig. 1 *H*, *arrowhead*).

Fredette et al. (1993) previously showed that PSA expression on the surface of myotubes in vivo was dramatically altered by neuromuscular activity blockade. Thus, while total NCAM expression was only modestly affected by activity blockade, PSA immunofluorescence was virtually abolished during all stages of muscle development. Since PSA was expressed on cultured myotubes at the time when they initiated spontaneous contractions (third day in culture) and because activity blockade prevented the upregulation of PSA in vivo, we decided to test more directly the possibility that electrical activity was regulating PSA. Thus spontaneous contractile activity was either completely blocked by bath application of TTX or increased by electrically stimulating 7-d-old myotubes (1 pulse/10 s; 24 h/d) for 72 h.

Chronic exposure to TTX did not affect myotube forma-

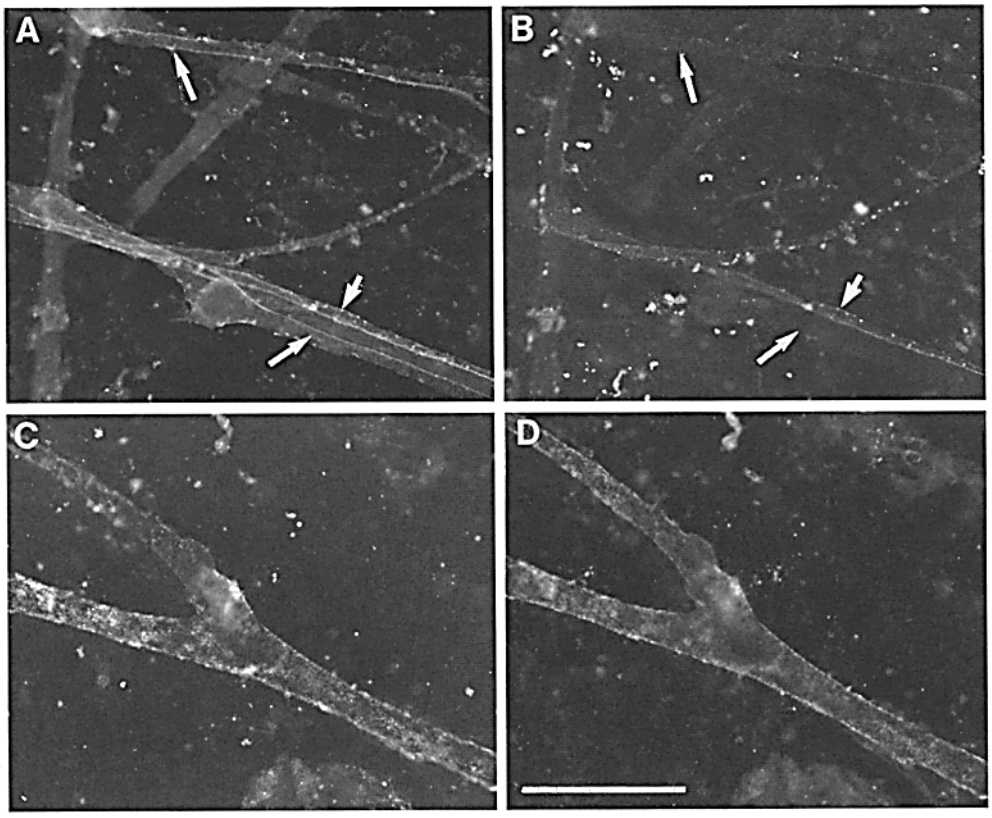

Figure 2. NCAM and PSA expression in the presence of TTX or electrical stimulation. 10 d myotube cultures grown in the presence of 10^{-6} M TTX for 8 d were stained for NCAM (Mab5E) (*A*) or for PSA (Mab5A5) (*B*). Similar cultures were electrically stimulated at a rate of one pulse every 10 s for 72 h, beginning on day 7, and stained for NCAM (*C*), or for PSA (*D*). TTX-treated myotubes (*A*) expressed similar levels of NCAM as electrically stimulated myotubes (*C*). However, 5A5 expression was diminished following TTX treatment (*B*) and many myotubes did not express PSA (*B*, *long arrows*). Some myotubes (*B*, *short arrows*) did exhibit PSA expression, but this was, in general, lower than that for electrically stimulated myotubes (*D*) or those with normal levels of spontaneous contractions. TTX-treated myotubes were also generally of smaller diameter than either control or electrically stimulated myotubes. Bar, 75 μm.

tion but did result in considerably thinner myotubes presumably because of the reduced synthesis of contractile proteins (Cerny and Bandman 1986). As occurred in vivo (Fredette et al., 1993), total NCAM was uniformly expressed on myotubes and the level of immunofluorescence was not visibly different between TTX (Fig. 2 *A*) and electrically stimulated (Fig. 2 *C*) myotubes. In contrast, PSA expression was very sensitive to myotube activity levels, being low to undetectable in myotubes whose activity had been blocked (Fig. 2 *B*) and very high in chronically stimulated myotubes (Fig. 2 *D*). PSA expression in these chronically stimulated myotubes was, in general, higher than in control spontaneously contracting myotubes.

Immunoblot Analysis of NCAM Isoforms during Secondary Myogenesis In Vitro

Since the mAb 5E does not distinguish among the different isoforms of NCAM it is possible that, although the overall expression of NCAM did not change during in vitro myogenesis, changes in the expression of individual isoforms may have occured during specific stages of muscle development. Previous studies in developing chick (Fredette et al., 1993; Yoshimi et al., 1993) and mouse muscles (Covault and Sanes, 1986) have shown that the 145-kD transmembrane isoform is expressed during early myogenesis whereas the 130-kD lipid-linked isoform becomes the predominant isoform later in development. However, since developing muscles in vivo are composed of a heterogeneous population of primary and secondary myoblasts and myotubes it is difficult to ascertain whether this in vivo switch in isoform expression occurs because NCAM isoform expression is regulated developmentally in all cell types or because different populations of muscle cells express different isoforms. For example, several in vitro studies concluded that myoblasts preferentially express the 145-kD transmembrane isoform while the 130-kD lipid-linked form is expressed on myotubes once fusion occurs (Moore et al., 1987; Knudsen et al., 1989; Tassin et al., 1991). A previous in vivo study from this lab (Fredette et al., 1993) observed that the 145-kD isoform predominated when muscles were composed of primary myotubes, with expression of the 130-kD isoform being associated with secondary myogenesis.

To clarify this issue, NCAM isoform expression was examined at several stages during in vitro secondary myogenesis. To determine the pattern of isoform expression the isoforms were examined with SDS-PAGE combined with immunoblot analysis. Cell cultures were harvested and homogenized at 1 d (homogenous culture of secondary myoblasts), 3 d (newly formed secondary myotubes), and 4–8 d (progressive maturation of myotubes) after plating. Half of the tissue homogenates were desialylated with neuraminidase to remove the polysialic acid associated with the NCAM isoforms.

The predominance of the different NCAM isoforms varied with the stage of myogenesis. In agreement with previous studies (Knudsen et al., 1989; Tassin et al., 1991), 1-d-old myoblast cultures contained NCAM of three different molecular weights when desialylated: 155, 145 and 130 kD,

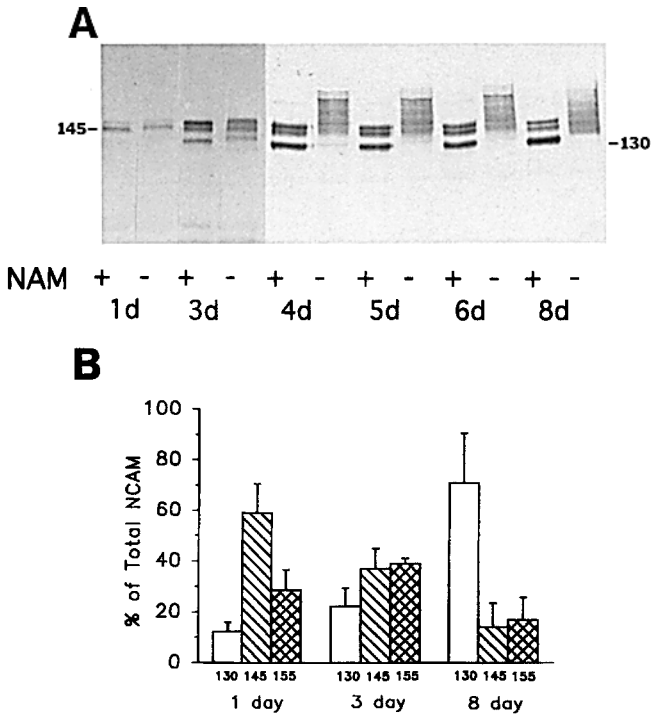

Figure 3. SDS gel electrophoresis and immunoblot analysis of NCAM isoforms and degree of polysialylation during muscle development in vitro. (*A*) Neuraminidase-desialylated proteins (+ lanes) from 1, 3, 4–8 d myotube cultures show that there are progressive changes in the proportions of the different NCAM isoforms during in vitro myogenesis. Myoblasts contain primarily the 145-kD isoform with lesser amounts of the 155-kD isoform. Weak expression of the 130-kD isoform, barely detectable on the immunoblot shown in *A* was confirmed with quantitative densitometry (see *B*). The 130- and 155-kD isoforms are up-regulated in newly formed, spontaneously contracting myotubes (*3d*). This pattern remained relatively constant until 8 d when the relative expression of the 155- and 145-kD isoforms decreased compared to the 130-kD isoform. NCAM from 1 and 3 d myotube culture homogenates that were not treated with neuraminidase was detected as three discrete bands (− lanes) indicating that at this stage of development NCAM was not polysialylated. In contrast, NCAM from 4 d myotubes was detected as a diffuse band running between 250–140 kD indicating that actively contracting myotubes have highly polysialylated NCAM. (*B*) Quantitative representation of the developmental changes in NCAM isoform expression. Densitometric scans were made of the NCAM immunoblots and the quantity of 155-, 145-, and 130-kD isoforms is expressed as a percent of the total NCAM present. Values are means ± SE from at least three separate experiments.

with the 145-kD isoform being most prominent and the 130-kD GPI-linked isoform being only weakly expressed (Fig. 3). Newly formed, spontaneously contracting, myotubes (3-d-old cultures) continued to express all three isoforms; however the expression of the 155-kD isoform had now increased sharply with respect to the 145-kD NCAM as did, to a lesser extent, the lipid-linked 130-kD isoform. 1-d later (4-d-old cultures), the expression of the 155- and 145-kD isoforms had remained relatively constant whereas the 130-kD NCAM had increased considerably. This pattern of NCAM expression remained relatively constant until day 8 when the relative expression of the 155- and the 145-kD isoforms decreased while that of the 130-kD NCAM increased to become the major isoform (Fig. 3, *A* and *B*).

The developmental regulation of PSA expression, shown previously with immunostaining, was also detectable on immunoblots. NCAM from 1- and 3-d-old muscle culture homogenates that were not treated with neuraminidase (Fig. 3 *A*, columns labeled with −) was detected as three discrete bands running at only slightly higher molecular weights than homogenates that were treated with neuraminidase (Fig. 3 *A*). In contrast, NCAM from 4 d and older cultures was detected as a diffuse band between 250–140 kD. These results indicate that the NCAM on secondary myoblasts and newly formed myotubes (i.e., 3 d) is not polysialylated while NCAM on older, actively contracting myotubes is highly polysialylated. These results agree with the PSA immunofluorescence which showed that staining with the mAb 5A5 was not detectable on 1-d-old myoblasts and was very weak on 2-d-old myoblasts while it was readily detectable on myotubes. These results also show that secondary myotubes growing in culture polysialylate all three major NCAM isoforms. This contrasts with chick muscle development in vivo, where Fredette et al. (1993) showed that the 145-kD isoform was not polysialylated at times when the 130 and 155 isoforms were. Although at present we do not have an explanation for these differences, it is possible that primary myotubes, which in vivo make a significant contribution to total muscle NCAM, sialylate isoforms differently than secondary myotubes.

Metabolic Labeling of NCAM Isoforms

The changes in NCAM isoform expression we observed could be due to differences in either the rate of synthesis of each isoform or their rate of turn-over. For example, it is possible that only myoblasts synthesize the 145-kD isoform and that its continued expression on myotubes is due to its slow rate of turn-over during myotube formation. Similarly, the switch in the predominant isoform observed by 8 d in culture may have resulted because by this stage of development the 155- and 145-kD isoforms were turning over more rapidly than the 130-kD isoform. Thus, if all were synthesized at the same rate, more of the 130-kD isoform would be present at any given time point.

To examine these possibilities [^{35}S]methionine pulse–chase experiments were performed on myotube cultures 4 and 7 d after plating. As shown in Fig. 4 *A*, all three isoforms are synthesized in 4-d-old myotube cultures indicating that the 145-kD isoform is not only synthesized by myoblasts as previously suggested (Covault et al., 1986; Moore et al., 1987) but also by myotubes which at this stage constitute the vast majority of the NCAM expressing cells in these cultures (Fig. 4 *A*). After 1 or 2 d of chase the level of [^{35}S]methionine labeling of all three isoforms of NCAM decreased at a similar rate. Optical density measurements (Fig. 4 *B*) indicated that the half life of all three isoforms at this stage of development was ~20 h.

The relative rates of synthesis of the three NCAM isoforms changed markedly between 4 and 7 d in culture (Fig. 4 *C*). By 7 d in culture the relative rate of synthesis of the 130-kD NCAM had greatly increased with respect to the 145- and 155-kD isoforms. Thus the predominance of the

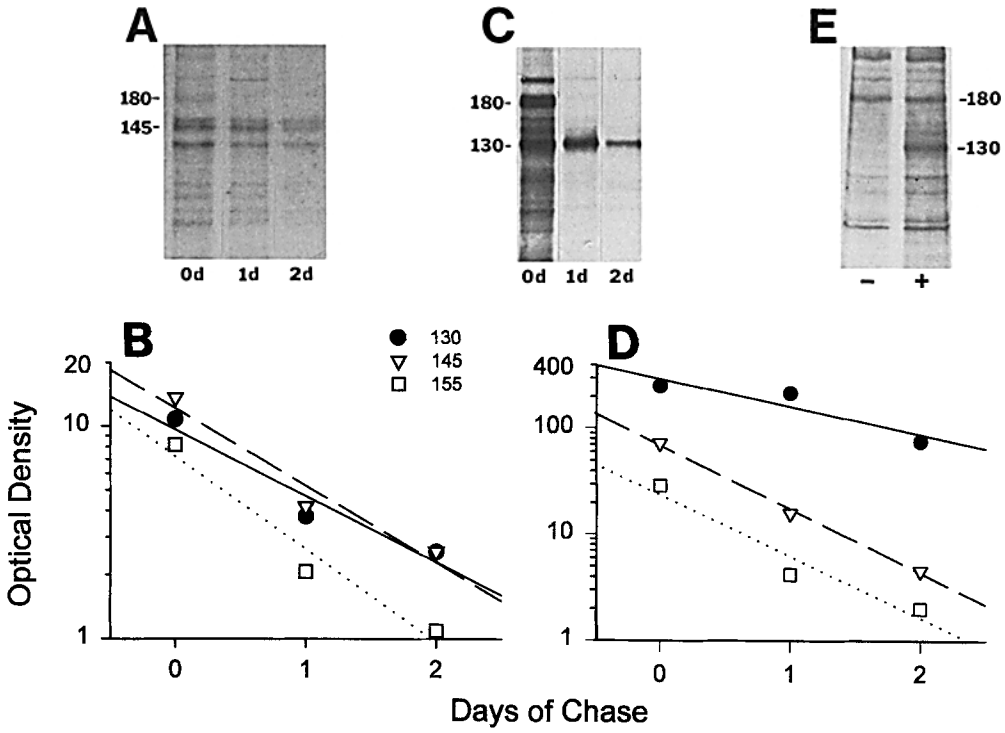

Figure 4. Rate of synthesis and turn-over of newly synthesized NCAM isoforms at 4 and 7 d in cultures. Myotube cultures were pulsed with [^{35}S]methionine for 8 h after which the proteins were either extracted immediately (*0d*) or chased with [^{35}S]methionine-free medium for 1 or 2 d. NCAM was immunoprecipitated and run on 6% SDS-PAGE gels (see Materials and Methods for additional details). (*A*) 4-d-old myotube cultures synthesize all three "muscle specific" isoforms with the level of synthesis of the 145 kD being slightly higher than the 155- and 130-kD isoforms. (*B*) Plotting the optical density measurements on semilogarithmic axes indicates that at this stage of muscle development all three isoforms have a half life of ~20 h. Note that there is a faint, but discernible band at 180 kD in the 0d lane. (*C*) While 7-d-old myotube cultures also synthesize all three "muscle specific" isoforms, the expression pattern dramatically differs from 4 d myotubes; the relative rate of synthesis of the 130-kD isoform is substantially higher than either the 155- and 145-kD isoforms. The level of expressison of all three isoforms progressively decreases following a 1 and 2d of chase. (*D*) Optical density measurements, plotted on semilogarithmic axes, indicates that the turn-over rate of the 130-kD isoform is significantly slower (half-life of 48 h) than both the 155- and 145-kD isoforms (both with half-life of 12 h). Also note that an intense 180-kD band can clearly be seen in the 0d lane but is absent following 1 and 2d of chase. (*E*) Not all NCAM isoforms synthesized in 8 d myotube cultures are polysialylated. [^{35}S]Methionine-labeled cultures show that the 130-kD isoform is detectable as a distinct band only when the homogenate is treated with neuraminidase (+) and that this band is completely absent in untreated samples (−). In contrast, the 180-kD isoform is clearly detectable as a discrete band of essentially the same molecular weight in both untreated (−) and treated (+) myotube homogenates.

130-kD isoform appears to be due, at least in part, to an increased rate of synthesis. However, pulse chase experiments also indicated that the turn-over rate of this isoform was slower compared to the 145- and 155-kD isoforms. From optical density measurements (Fig. 4 *D*) its half life was ~48 h compared to 12 h for both the 145- and 155-kD isoforms. Thus, the relative abundance of the GPI-linked 130 isoform in more mature myotubes appears to be due to both an increase in its relative rate of synthesis and a decrease in its rate of degradation.

In addition, in both 4 and 7 d myotube cultures we noticed a band in the position where the 180-kD NCAM isoform would appear. The 180-kD isoform has been considered to be nerve specific (but see Tassin et al., 1991; Lyons et al., 1992), and in most immunoblots of cultured myotubes, there was only a very faint band evident in this position (see for example Fig. 3 *A*, 4–6 d cultures). Nevertheless, in the autoradiograms from 8 d cultures (Fig. 4 *C*, *0 d*), the protein running at this position was being synthesized at a higher rate than any of the other NCAM isoforms. However, unlike the 155-, 145-, and 130-kD NCAM isoforms, this protein had a very fast turn-over rate, with a half-life of less than 8 h, since it was virtually absent following a 1 d chase (Fig. 4 *C*, 1 d). In striking contrast to the other three isoforms, the 180-kD NCAM was not polysialylated as indicated by the distinct band running at 180-kD from the 8-d-old culture homogenate that was not treated with neuraminidase (Fig. 4 *E*).

A protein with a very high molecular weight (>200 kD) was frequently immunoprecipitated along with NCAM using 5E-conjugated Sepharose beads (for example see Figs. 4 *C* and 7 *B*). While the identity of this protein is not known, it is clearly not NCAM since it was not recognized by either 5E monoclonal antibody (Figs. 3 and 5) or by polyclonal anti-NCAM (data not shown). Preliminary experiments indicate that some, but not all, of the material running at this position is myosin that bound nonspecifically to the beads used in the immunoprecipitation.

Expression of a 180-kD NCAM Isoform by Skeletal Muscle Cells

Metabolically labeled myotubes synthesized an apparent NCAM isoform with a molecular weight of 180 kD (Fig. 4). Previous studies had indicated that this transmembrane NCAM isoform, with a large intracellular domain, was exclusively synthesized in neuronal tissue, and was absent from skeletal muscle (but see Tassin et al., 1991). To better characterize the 180-kD isoform of NCAM synthesized in muscle, homogenate extracts were prepared from St. 37 chick hindlimb muscles, 5 d myotube cultures, and from sciatic nerve. Immunoblots were stained with either a

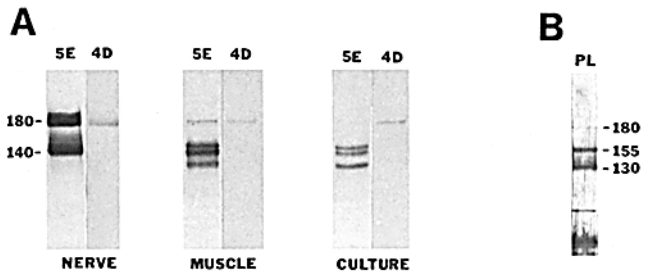

Figure 5. The "neuronal" 180-kD NCAM isoform is expressed in developing chick muscle, both in vivo and in vitro. Neuraminidase-desialylated proteins, extracted with NP-40 from St 37 sciatic (*NERVE*), hindlimb muscles (*MUSCLE*) and 5d myotube cultures (*CULTURE*) were separated by SDS-PAGE and immunoblotted with either an mAb that recognizes all NCAM isoforms (5E) or with an mAb that specifically recognizes only the 180-kD isoform (4D). The 4D mAb specifically recognized a 180-kD isoform in embryonic nerve, muscle and myotube culture extracts indicating that this isoform is not exclusively expressed by neuronal tissue. Immunoprecipitated NCAM was separated by SDS-PAGE and incubated first with PNA, and then immunoblotted with ant-PNA. Peanut lectin (*PL*) bound to 180-, 155-, and 130-kD NCAM isoforms, but not to the 145-kD isoform indicating that this isoform does not contain the MSD region.

mAb that recognized all NCAM isoforms (5E) or with one specific for the 180-kD neuronal isoform (4D). Sciatic nerve extracts from St 37 chick embryos contained both the 140- and 180-kD NCAM isoforms (Fig. 5, *5E, NERVE*) and in agreement with Watanabe et al. (1986), the 4D mAb exclusively recognized the 180-kD isoform (Fig. 5, *4D, NERVE*). The predominant NCAM isoforms observed in extracts from St 37 chick muscles were the 130-, 145-, and 155-kD isoforms; nevertheless the 180-kD isoform was clearly detectable (Fig. 5, *5E, MUSCLE*; see also Fredette et al., 1993) and recognized by the 4D mAb (Fig. 5, *4D, MUSCLE*). To rule out the possibility that the 180-kD NCAM isoform observed in the muscle extracts was due to NCAM derived from intramuscular nerves, myotube cultures were homogenized and analyzed by immunoblotting with either the 5E (Fig. 5, *5E, CULTURE*; see also Fig. 3) or 4D (Fig. 5, *4D, CULTURE*) mAbs. As observed in the muscle extracts, cultured myotubes also contained the 180-kD isoform of NCAM.

The rate of synthesis of the 180-kD isoform in myotube cultures increased developmentally, being greater at 7 d than at 5 d (Fig. 4, *A* and *C*). In addition, its turn-over rate was significantly faster than that of the other isoforms since the metabolically labeled 180-kD isoform was completely absent with only 1 d of chase (Fig. 4 *C*). This high rate of turn over may explain why the 180-kD isoform is only weakly detected in immunoblot analysis (Fig. 3).

Specific NCAM Isoforms Contain a Peanut Lectin Binding Region

The 155- and 125-kD isoforms of NCAM from the C2 skeletal muscle cell line contain a muscle-specific domain (MSD) splice insert which contains an O-linked glycosylation site that is recognized by peanut lectin (Walsh et al., 1989). To characterize the binding of peanut lectin to NCAM from chick skeletal muscle, NCAM was immunoprecipitated from 5-d-old secondary myotube cultures, treated with neuraminidase, separated by SDS-PAGE, and transferred onto Immobilon-P membranes (see Materials and Methods for details). Binding of peanut lectin was detected on the 155- and 130-kD isoform bands but not on the 145-kD band, indicating that the latter isoform lacks the MSD insert (Fig. 5, *PL*) (see also Yoshimi et al., 1993). Peanut lectin also bound to the 180-kD "nerve" specific NCAM isoform, indicating that this isoform also contains the MSD. Byeon et al. (1995) recently found that in embryonic chick cardiac muscle the 125-, 155-, and 185-kD NCAM isoforms contained the MSD insert, while the 145- and 180-kD isoforms did not.

Molecular Mechanisms Regulating the Synthesis of NCAM Isoforms

Total NCAM is gradually down-regulated along the surface of embryonic muscle fibers following innervation, declining around the time of hatching in chicks (Tosney et al., 1986) and of birth in mammals when it becomes confined mainly to neuromuscular junctions (Covault and Sanes, 1985). However, if neuromuscular activity is decreased in adult muscles by denervation (Covault and Sanes, 1985, 1986; Covault et al., 1986) or TTX treatment (Covault and Sanes, 1985) there is a rapid up-regulation of NCAM. These results show that the overall expression of NCAM on neonatal and adult muscle fibers is regulated by neuromuscular activity. However, at earlier stages of embryonic development when overall NCAM synthesis was not altered by activity blockade (Fredette et al., 1993), the synthesis of specific isoforms was. How might electrical activity be coupled to isoform synthesis?

Muscle contraction involves a series of events including depolarization of the sarcoplasmic reticulum and the t-tubules, activation of voltage-sensitive Ca^{2+} channels, release of Ca^{2+} from the sarcoplasmic reticulum (SR), Ca^{2+}-dependent activation of actin and myosin cross-bridges, and finally the reuptake of Ca^{2+} back into the SR. Increased levels of intracellular Ca^{2+} are known to be a critical element in intracellular signaling for many different cell types. In muscle fibers, neurally evoked Ca^{2+} release is believed to mediate changes in the synthesis of macromolecules including ACh receptors, myosin heavy chains, and Ca^{2+} ATPases (for general review see Pette and Vrbova, 1992).

To test whether Ca^{2+} plays a role in regulating NCAM isoform expression, one group of 5-d-old myotube cultures was treated with the Ca^{2+} channel blocker verapamil for 2 d, and then homogenized for immunoblot analysis. The other group served as a control. At the time this 7 d culture was analyzed, the 155- and 130-kD isoforms had already been up-regulated, becoming the predominant isoforms, while the 145-kD isoform had been significantly down-regulated (Fig. 6 *A*, lane *1*). In contrast, when voltage-activated Ca^{2+} channels were chronically blocked with verapamil (Fig. 6 *A*, lane *2*), the 145-kD isoform (*arrow*) now became the predominant isoform. Thus, preventing the entry of Ca^{2+} resulted in a less mature isoform pattern relative to the control, suggesting that Ca^{2+} entry during spontaneous electrical activity may serve as the first step in triggering the developmental isoform switch we observed.

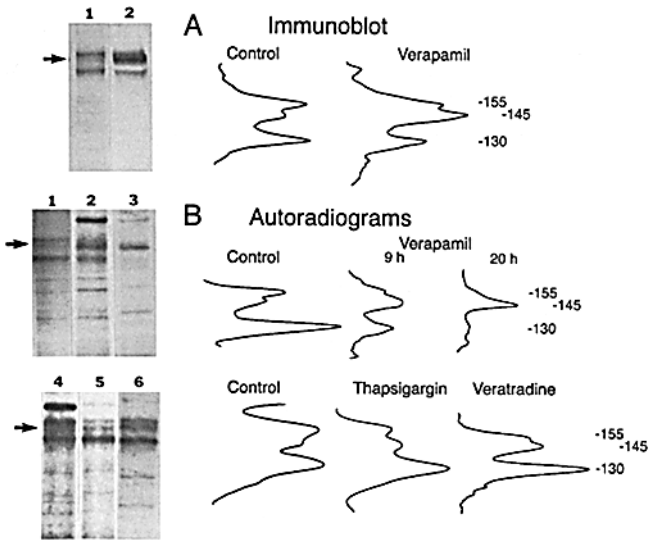

Figure 6. Chronic blockade of voltage-operated Ca^{2+} channels results in a dramatic change in NCAM isoform expression. (*A*) Immunoblot analysis of NCAM from desialylated proteins extracted from 7 d control myotube cultures (lane *1*) and cultures treated for 24 h with verapamil (10^{-6} M) (lane *2*) shows that blockade of Ca^{2+} channels results in a dramatic increase of the 145-kD NCAM isoform relative to the 130- and 155-kD isoforms. (*B*) Autoradiograms and optical density scans of 5-d-old cultures pulsed with [^{35}S]methionine show that chronic verapamil treatment results in a dramatic decrease in the synthesis of the 130- and 155-kD isoforms of NCAM. This effect can be seen after only a 9 h verapamil treatment (lane *2*) but is significantly more dramatic after a 20 h treatment (lane *3*). 5 d cultures treated for 24 h with thapsigargin (10 mM) (lane *5*) or veratridine (10^{-7} M) (lane *6*), which would increase intracellular Ca^{2+}, decreased the relative expression of the 155-kD isoform with respect to the 130-kD isoform.

To determine the extent to which this altered isoform expression pattern was due to altered synthesis of individual isoforms, 5-d-old cultures were treated with verapamil for 1 or 12 h prior to an 8 h pulse with [^{35}S]methionine. Verapamil was maintained in the medium for the duration of the pulse. Untreated 5-d-old cultures (Fig. 6 *B*, lane *1*) synthesized all three isoforms, with the 155- and 130-kD isoforms being synthesized at a relatively higher rate compared to the 145-kD isoform. This was similar to the relative amounts of these isoforms detected in immunoblots, such as that shown in Fig. 6 *A*. After a brief 1 h pre-treatment with verapamil (9 h total treatment), the 145-kD isoform (*arrow*) was now increased markedly with respect to the 155- and 130-kD isoforms. The effect was more dramatic following a 12 h pre-treatment with verapamil (20 h total); in this case (Fig. 6 *B*, lane *3*), synthesis of the 155- and 130-kD isoforms was completely inhibited, with only the 145-kD isoform still being synthesized.

Intracellular Ca^{2+} levels can be increased with thapsigargin, a drug that specifically inhibits the endoplasmic reticulum Ca^{2+}-ATPase (Thastrup et al., 1990). As can be seen in Fig. 6 *B* (lane *5*) a 24 h treatment with this drug, had the opposite effect of verapamil, producing a more mature isoform pattern, in which the 130-kD predominates with lower rates of synthesis of both the 145- and 155-kD isoforms. Thus these cultures, which were 6-d-old at the time of the analysis, had an isoform expression pattern more like control 8 or 9 d cultures (see for example the immunoblots in Fig. 3 *A* and the autoradiogram in Fig. 4 *B*). The Na^+ channel activator veratradine also produced a more mature isoform pattern (Fig. 6 *B*, lane *6*). When this drug was added to the cultures it produced more frequent spontaneous contractions, and thus may have indirectly caused increased entry of Ca^{2+}.

The amount of the 180-kD isoform synthesized varied between control cultures of the same age (compare lanes *1* and *4* in Fig. 6). This could be caused by differences in the level of spontaneous electrical activity which varies between cultures. In support of this possibility, older, more actively contracting myotubes consistently synthesized more of the 180-kD NCAM than younger less active myotubes (for example see Fig. 4).

Previous studies have indicated that the 180-kD NCAM isoform is a substrate for endogenous Ca^{2+}-activated proteolysis (Covault et al., 1991; Sheppard et al., 1991). Pulse–chase experiments presented in this study show that the 180-kD isoform has a very rapid rate of turn-over (Fig. 4). Whether this is due to Ca^{2+}-activated proteolysis is not known. However, consistent with this possibility, blockade of voltage-activated Ca^{2+}channels with verapamil, which would be expected to lower intracellular Ca^{2+}, resulted in an increase in the amount of the 180-kD isoform (compare lanes *1* and *2* in Fig. 6 *B*). Conversely, thapsigargin or veratradine, both of which increase the level of intracellular Ca^{2+}, caused a decrease in the amount of 180-kD NCAM (compare lanes *5* and *6* with lane *4* in Fig. 6 *B*).

In conclusion, it appears that Ca^{2+} entry via voltage dependent Ca^{2+} channels has the effect of increasing the synthesis of the 155- and 130-kD isoforms relative to that of the 145-kD form. Since these different NCAM isoforms arise by alternative splicing from a single gene, the entry of Ca^{2+} via voltage sensitive channels appears to be the first step in the pathway by which electrical activity controls the developmentally regulated isoform switch we have described. We therefore decided to explore possible downstream consequences of this intial step.

Another molecule, whose synthesis is known to be regulated by electrical activity, is the AChR. Synthesis of this receptor in nonsynaptic nuclei is down-regulated following initial innervation and activation of myotubes, but is rapidly restored following denervation (Berg et al., 1972; Miledi and Potter, 1971). More recent studies have implicated entry of Ca^{2+} and activation of PKC in this down-regulation. Denervation of chick hind limb muscles (Huang et al., 1992) or blockade of spontaneous contractions in cultured myotubes with TTX (Klarsfeld et al., 1989; Mendelzon et al., 1994) causes a dramatic decrease in nuclear PKC activity and an increase in the level of AChR mRNA. In addition, inhibition of PKC activity with selective inhibitors leads to an increase in AChR mRNA synthesis in both TTX-treated cultured myotubes (Fontaine et al., 1987; Klarsfeld et al., 1989) and in denervated chick muscles (Huang et al., 1992). Since the developmentally regulated NCAM isoform switch we observed was also sensitive to activity and appeared to require entry of Ca^{2+}, we examined the possible involvement of PKC in this regulation.

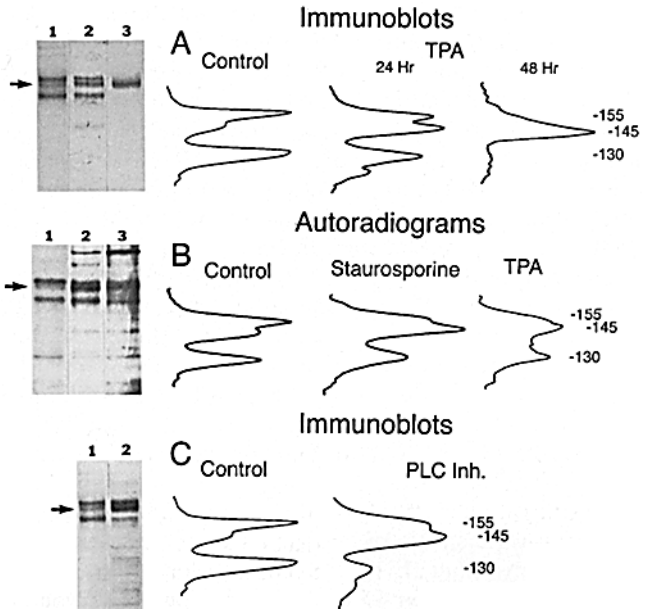

Figure 7. Chronic inhibition of PKC results in a dramatic change in NCAM isoform expression. (*A*) Immunoblots and optical density scans of NCAM from desialylated proteins extracted from 7 d control myotube cultures (lane *1*) and cultures treated for 24 h (lane *2*) and 72 h (lane *3*) with TPA (10^{-6} M) (lane *2*) shows that inhibition of PKC results in a significant increase in the 145-kD NCAM isoform relative to the 130- and 155-kD isoforms. Synthesis of NCAM isoforms following treatment with staurosporine or long-term TPA. Control 5 day cultures show that synthesis of the 145-kD isoform (lane *1*, *arrow*) is lower than that of the 155- and 130-kD isoforms. 5-d-old cultures treated for 20 h with staurosporine (10^{-7} M) (lane *2*) or long-term TPA (10^{-6} M) (lane *3*) resulted in increased synthesis of the 145-kD isoform (*arrow*) relative to the other two isoforms. (*C*) Inhibition of phosphatidylcholine metabolism with D609 (30 mM) (lane *4*) mimics the effects seen with chronic TPA treatment.

5-d-old myotube cultures were treated with the phorbol ester, TPA (10^{-6} M), for 24 or 72 h and subsequently homogenized to assess NCAM expression using SDS-PAGE combined with immunoblot analysis. Continuous application of this concentration of TPA has previously been shown to substantially deplete PKC in nuclear fractions from chick muscle fibers after 16 h of treatment (Huang et al., 1992). After a 24 h treatment with TPA the expression pattern of NCAM isoforms on cultured myotubes differed significantly from untreated control cultures. The control cultures of this stage, the level of the 145-kD NCAM was substantially lower than the 155- and 130-kD isoforms (Fig. 7 *A*, lane *1*, *arrow*), whereas in the cultures treated with TPA for 24 h, all three isoforms were being expressed at approximately equivalent levels (Fig. 7 *A*, lane *2*). Thus it appeared that the 145-kD isoform had been significantly increased relative to the other two isoforms. This effect was even more obvious following a 72 h treatment when the 145-kD isoform was the only isoform detected (Fig. 7 *A*, lane *3*). Similar results were obained when relative rates of isoform synthesis were measured in cultures pulsed with [^{35}S]methionine following treatment with staurosporine or long-term TPA treatment, both of which would be expected to down-regulate PKC. As seen in Fig. 7 *B* (lanes *1–3*), both of these treatments increased the synthesis of the 145-kD isoform relative to the other two isoforms.

In summary, blocking Ca^{2+} entry via voltage-dependent Ca^{2+} channels or inhibiting PKC appeared to selectively inhibit the synthesis of the 130- and 155-kD isoforms while increasing that of the 145-kD isoform.

PKC is activated by Ca^{2+} and diacylglycerol following hydrolysis of phosphatidylinositol and/or phosphatidylcholine. Hydrolysis of the latter is believed to result in a more sustained elevation in diacylglycerol which consequently sustains the activation of PKC (Asaoka et al., 1992). The phospholipid that supplies diacylglycerol during neuromuscular activity is not known. However, studies on *Ascaris suum* muscles have shown that ACh stimulates phosphatidylcholine metabolism (Arevalo and Saz, 1992). To determine whether sustained activation of PKC, via the phosphatidylcholine pathway, is required for the developmental switch in NCAM isoform expression 5-d-old myotube cultures were treated for 48 h with a selective inhibitor of phosphatidylcholine-specific phospholipase C (D609). As was observed with long-term TPA treatment, inhibition of PKC activation with D609 altered the pattern of NCAM isoform expression such that the 145 kD became the predominant isoform of NCAM (Fig. 7 *B*).

Molecular Mechanisms Regulating the Polysialylation of NCAM

As shown above, electrical activity appears to control the alternative splicing of NCAM isoforms via influx of Ca^{2+} and activation of PKC. Since activity was also shown to be required for the polysialylation of NCAM, we explored the possibility that this might be mediated by a similar intracellular signaling pathway. Although not as extensively investigated as the isoform switch, our data is consistent with this possibility. As shown in the immunoblot in Fig. 8 *A*, untreated NCAM from 4 d cultured myotubes ran as diffuse smear (lane *1*). Treatment with the PSA specific endoneuraminidase (lane *2*) altered the mobility of that portion of the NCAM running above the indicated line, which we operationally define as polysialylated NCAM. As shown in Fig. 8 *B*, a 24 h treatment with TPA (lane *3*) reduced the proportion of the NCAM running above this line as compared to the control (lane *1*), as did a 24 h treatment with verapamil (lane *5*). Correlated with this, discrete bands, representing nonsialylated NCAM, became discernable in the untreated sample (Fig. 8, indicated by *stars* in lanes *3* and *5*). Verapamil treatment for 24 h was more effective than 24 h of TPA, both in reducing polysialylation and bringing about the isoform switch (compare lanes *2*, *4*, and *6*). This would be expected since verapamil would have blocked Ca^{2+} entry for the full 24 h while PKC activity is only reduced by long term TPA after ~16 h (Huang et al., 1992). The autoradiogram in Fig. 8 *C* shows that the NCAM being synthesized in the verapamil treated cultures at the 24 h time point, is all of the 145-kD isoform and that it is not sialylated (the mobility of the discete band marked by the *star* in the untreated sample of lane *1* is not altered by neuraminidase treatment, lane *2*). In summary, as was shown for the isoform switch, electrical activity appears to regulate NCAM polysialylation via

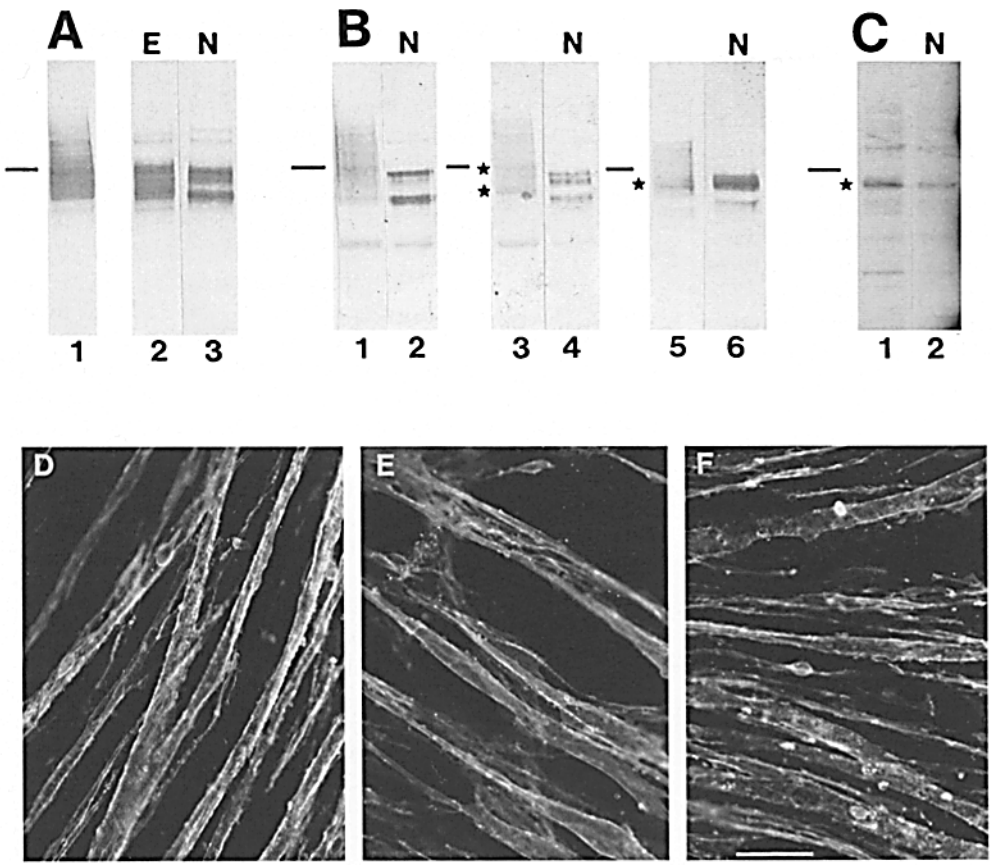

Figure 8. Expression of PSA and total NCAM on cultured myotubes treated with TPA or verapamil. (*A*) NCAM immunoblots, from 4 d myotube cultures, that were untreated (lane *1*) or treated with endoneuraminidase (lane *2*) or neuraminidase (lane *3*). The NCAM running above the line in lane *1* had its mobility altered following treatment with the PSA specific endoneuraminidase (lane *2*) and thus represents the polysialylated NCAM. (*B*) Immunoblots from similar cultures that were untreated (lanes *1* and *2*) or treated for 24 h with TPA (lanes *3* and *4*) or verapamil (lanes *5* and *6*). After 24 h of treatment with TPA (lane *3*), the proportion of polysialylated NCAM, running above the line, is reduced compared to the control (lane *1*). In addition distinct bands representing 145/155-kD and the 130-kD NCAM (indicated by *stars*) become visible even without neuraminidase treatment. The switch from the 155- and 130-kD isoforms toward the 145-kD isoform described earlier is also seen (compare lanes *2* and *4*). After treatment with verapamil for 24 h both the reduction in the proportion of polysialylated NCAM (lane *5*) and the switch toward expression of the 145-kD isoform, indicated by the star (lanes *5* and *6*), is more complete (see text for additional explanation). (*C*) An audoradiograph from a similar culture treated for 24 h with verapamil, shows that the metabolically labeled NCAM being synthesied at this time (lane *1*, without neuraminidase treatment) is almost entirely the 145-kD isoform and is not sialylated (the mobility of a distinct band at this position is identical in the untreated (lane *1*) and neuraminidase treated (lane *2*) sample. (*D–F*) Cultures immunostained to visualize total NCAM expression. 4 d myotube cultures were either untreated (*D*) or treated for 24 h with verapamil (16 μM) (*F*) or TPA (10^{-6} M) (*E*) and immunostained with mAb 5E that recognizes all NCAM isoforms. NCAM levels and distribution were generally similar in all cases. Overall, myotube morphology was also similar except in the TPA treated cultures where some of the myotubes appeared to be more flattened and less uniformly cylindrical. *E*, endoneuraminidase; *N*, neuraminidase. Bar, 15 μm.

an intracellular signalling pathway involving Ca^{2+} influx and PKC.

Long-term treatments with verapamil or TPA produced dramatic alterations in the proportion of the different NCAM isoforms expressed and in their level of sialylation. Nevertheless, myotubes in such cultures remained healthy in appearance and retained a typical myotube morphology. In addition, immunostaining with mAb 5E, revealed that both TPA (Fig. 8 *E*) and verapamil (Fig. 8 *F*) treated myotubes continued to express NCAM with a level and distribution similar to controls (Fig. 8 *D*).

Discussion

Expression and Regulation of NCAM and PSA during In Vitro Myogenesis

The present study confirms the results of Mege et al. (1992) showing that NCAM is strongly and uniformly expressed by myogenic cells throughout in vitro myogenesis. NCAM is prevalent on cultured myoblasts and, unlike N-cadherin (Hahn and Covault, 1992; Mege et al., 1992), is not down-regulated in older, spontaneously contracting myotubes. This temporal pattern of expression is similar to that observed during in vivo development of chicken muscle, in which N-cadherin is rapidly down-regulated (Fredette et al., 1993; Hahn and Covault, 1992), while NCAM continues to be expressed for at least a week after muscle fibers begin to be electrically activated by neurons (Bekoff et al., 1975; O'Donovan and Landmesser, 1987). NCAM declines only around the time of hatching in the chick (Tosney et al., 1986) and birth in the mouse (Covault and Sanes, 1985).

In contrast, we found that the polysialylation of NCAM was developmentally regulated during secondary myogenesis in vitro and that this was controlled by electrical activity. Previous observations had indicated that PSA synthesis by myotubes in vivo was either directly or indirectly regulated by neuromuscular activity since activity blockade by dTC prevented its usual up-regulation during secondary myogenesis (Fredette et al., 1993). However these results might have been explained by some direct effect of

dTC acting on either classical skeletal muscle α1 or "neuronal" α7 AChRs, which are both present on chick myotubes at early developmental stages (Corriveau et al., 1995). The present results show that it is indeed blockade of electrical activity and not some pharmacological effect of dTC that prevents PSA expression. Specifically, the level of PSA immunostaining increased sharply when newly formed myotubes began to spontaneously contract. Furthermore, it was greatly reduced in myotube cultures treated with TTX to block spontaneous contractions and was increased in cultures that were electrically stimulated to increase the frequency of contractions. Together, the present results provide strong support for the hypothesis that neuromuscular activity is an important factor regulating PSA synthesis in muscle during development. Interestingly, not all cultured myotubes expressed PSA even though they were well formed and stained intensely for total NCAM. Upon visual inspection of the cultures, it was clear that some myotubes were not spontaneously contracting nor could they be induced to contract with electrical stimulation. It is therefore possible that the myotubes that did not express PSA were the same ones that did not exhibit spontaneous contractile activity.

It is not yet known how neuromuscular activity regulates NCAM polysialylation. Recently, Bruses et al. (1995) found that PSA expression in developing chick ciliary ganglia appeared to be regulated at the level of a polysialyltransferase whose activity correlated well with temporal patterns of PSA expression. It is possible that polysialyltransferase activity also regulates PSA synthesis in developing muscle such that the absence of PSA on proliferating myoblasts and newly formed myotubes is because these cells do not yet contain sufficient levels of active polysialyltransferase. If PSA synthesis by muscle is shown to be regulated at the level of the polysialyltransferase, our overall results would strongly suggest that either the synthesis or the activity of the relevant sialyltransferases is controlled, at least in part, by electrical activity, and that the intracellular signaling pathway involves Ca^{2+} influx and PKC.

Interestingly, while mature myotubes in culture synthesize four different isoforms of NCAM not all are polysialylated. In contrast to the other isoforms, the 180-kD isoform was not polysialylated even though it was the predominant form being synthesized (Fig. 4 *E*). How such selective isoform polysialylation occurs is not known. Several studies have reported selective sialylation of different NCAM isoforms in both brain (Breen et al., 1987; Breen and Regan, 1988; Bartsch, 1990), and in developing chick muscle (Fredette et al., 1993). However, both brain and developing muscle are highly heterogeneous with respect to the type of cells they contain and their stage of development (i.e., myoblasts vs. myotubes and/or primary vs. secondary myotubes). It is thus possible that the sialylated and nonsialylated isoforms observed in the above studies were being synthesized by different cell types. However, the present observations strongly suggest that an individual cell is capable of preferentially polysialylating different isoforms, since in the developmentally homogeneous population of myotubes in our cultures the 130-, 145-, and 155-kD isoforms were polysialylated while the 180-kD isoform was not.

Expression of NCAM Isoforms during Myogenesis In Vitro

We have shown in the present study that the expression of specific NCAM isoforms is temporally regulated during chick muscle development in vitro, generally confirming a number of earlier studies (Covault et al., 1986; Moore et al., 1987; Tassin et al., 1991). Several of these studies, using both primary cultures of muscle cells (Knudsen et al., 1989; Tassin et al., 1991; but see Yoshimi et al., 1993) and muscle cell lines (Moore et al., 1987), reported that myoblasts express only the 145-kD isoform while the 130-kD isoform is expressed by myotubes at the time of fusion. However, it is clear from our metabolic labeling results that the synthesis of the 145-kD NCAM is not restricted to myoblasts since both newly formed and mature myotubes in culture continue to synthesize the 145-kD as well as the 155- and 130-kD isoforms. In addition, following treatments with drugs that caused our cultures to express primarily the 145-kD isoform (for example, long-term TPA), immunostaining revealed that this NCAM isoform was distributed uniformly in well-formed myotubes and was not concentrated on nor confined to myoblasts.

Earlier studies of NCAM isoform expression during heart (Hoffman et al., 1990) and skeletal muscle (Fredette et al., 1993) development considered the 180-kD isoform to be of neural origin (however see Tassin et al., 1991; Lyons et al., 1992). The present study, however, indicates that the 180-kD isoform is indeed expressed by developing skeletal muscles. Very recent observations have shown that developing heart muscle expresses both 180- and 185-kD isoforms of NCAM with the latter containing the MSD insert (Byeon et al., 1995). The present metabolic labeling studies of myotube cultures indicate that the 180-kD is also synthesized at a high rate.

Regulation of NCAM Isoform Expression during In Vitro Myogenesis

During avian muscle development in vivo (Covault et al., 1986; Fredette et al., 1993; Yoshimi et al., 1993), and in vitro (this study; Covault et al., 1986; Tassin et al., 1991; Yoshimi et al., 1993; Lyles et al., 1993), the predominantly expressed NCAM isoform switches from the transmembrane 145-kD to the GPI-linked 130-kD isoform. In vivo, this developmental switch, due to alternative splicing, appears to be regulated by neuromuscular activity since it does not occur if activity is chronically blocked (Fredette et al., 1993).

The present study has begun to define the signaling events that link electrical activity at the cell surface to the regulation of NCAM alternative splicing. Specifically we have found that an increase in intracellular Ca^{2+} levels and subsequent activation of PKC appear to regulate this developmental isoform switch, at least in cultured myotubes. Blockade of Ca^{2+} influx through voltage sensitive Ca^{2+} channels by verapamil, or inhibition of PKC by staurosporine or long-term TPA treatment increased the synthesis of the 145-kD isoform, while decreasing the synthesis of both the 155- and the 130-kD isoforms. In contrast, treating with drugs that presumably would increase intracellular Ca^{2+} levels, such as veratradine, (a sodium channel ac-

tivator that increased the frequency of spontaneous myotube contractions), or thapsigargin (a Ca^{2+}-ATPase inhibitor that prevents the resequestration of cytoplasmic Ca^{2+}) increased the synthesis of the 130-kD NCAM.

The observations that we and others have made show striking similarities between the regulation of NCAM and the AChR. The level of expression of both AChRs (Betz and Changeux, 1979) and NCAM (Covault and Sanes, 1986) is down-regulated by neuromuscular activity and dramatically up-regulated following activity blockade (Covault and Sanes, 1985). This up-regulation of both AChRs and NCAM is prevented by electrically stimulating the denervated muscle fibers (Lomo and Westgaard, 1975; Goldman et al., 1988; Covault and Sanes, 1985). Several studies have proposed that Ca^{2+} and PKC are involved in a second messenger cascade that couples membrane depolarization with transcriptional factors that regulate the synthesis of the AChR. Membrane depolarization causes a transient increase in intracellular Ca^{2+} and translocation of PKC (Richter et al., 1987) while blocking depolarization causes a decrease in nuclear PKC activity (Huang et al., 1992; Mendelzon et al., 1994). Blockers of voltage-activated Ca^{2+} channels increase the expression (Shainberg et al., 1976) and synthesis (Klarsfeld et al., 1989; Huang et al., 1994) of AChRs in muscle cultures. Similarly, inhibition of PKC increases AChR α subunit synthesis in cultured myotubes (Klarsfeld et al., 1989).

The precise mechanisms by which Ca^{2+} ions and PKC regulate alternative splicing of NCAM during muscle development are not understood. Recently, Huang et al. (1993) showed that the myogenic factor, myogenin, declines rapidly in electrically stimulated denervated chick muscle at a rate comparable to the decline in AChR gene activation. They also showed that myogenin gene transcription was blocked by PKC inhibitors. One possible explanation for the regulation of NCAM isoforms is that membrane depolarization leads to both an increase in intracellular Ca^{2+} and translocation of the Ca^{2+}-dependent PKC into the nucleus where it phosphorylates myogenic transcription and/or splicing factors which in turn regulate the alternative splicing of NCAM. Mendelzon et al. (1994) recently proposed that the repression of AChR biosynthesis by membrane depolarization results from the phosphorylation of myogenin via the PKC second messenger pathway. Phosphorylation of myogenin inhibits its binding to DNA which in turn prevents the transcription of the AChR (Mendelzon et al., 1994). Whether myogenin or other myogenic transcription factors are involved in alternative splicing of NCAM is currently under investigation.

NCAM, PSA, and Neuromuscular Development

The precise roles played by PSA and the different isoforms of NCAM in promoting skeletal myogenesis and synapse formation are not known. Since the gross locomotor capabilities of NCAM-deficient mice appear to be normal (Cremer et al., 1994), NCAM and PSA do not seem to be essential for many aspects of neuromuscular development. However, it is possible that other cell adhesion molecules compensated for the loss of NCAM and PSA in these animals. For example, while PSA is unique to NCAM, its proposed function in reducing some of the molecular interactions involved in adhesion/signaling could be compensated for by a number of other molecular alterations that also affect adhesion or signaling. Alternatively, several different molecular interactions may contribute to ensure that a specific developmental process takes place, but either alone may suffice. For example, the separation of secondary myotubes from clusters is associated with both a down regulation of N-cadherin and an up-regulation of PSA, both of which would reduce adhesion. However, either one of these alterations in adhesion molecule expression may be sufficient for separation to occur. Thus PSA expression would only become critical for this process in situtations where the down regulation of N-cadherin failed to occur. Finally, more detailed observations of these NCAM-deficient mice may reveal alterations in neuromuscular development that are not detectable at the behavioral level, or alternately that are compensated for by subsequent developmental processes.

A number of observations do indicate that NCAM and PSA are significantly involved in myogenesis and neuromuscular development. Fusion of C2 myoblasts was enhanced when they were transfected with a human gene construct for either the GPI-linked (Dickson et al., 1990) or the 140-kD transmembrane (Peck and Walsh., 1993) NCAM isoform. In contrast, removal of the GPI-linked isoform from myoblasts with phospholipase C retarded myotube fusion (Knudsen et al., 1989). In vivo, injection of anti-NCAM reduced the number and length of intramuscular nerve branches (Landmesser et al., 1988) and PSA removal resulted in a reduction in both neuromuscular synapse formation and motoneuron survival (Tang and Landmesser, 1993). Finally, activity blockade, which prevents the expression of PSA on myotubes, but not nerve, interfered with the separation of myotubes from myotube clusters (Fredette et al., 1993). In the adult, other studies provide evidence that NCAM promotes nerve terminal sprouting following localized paralysis (Booth et al., 1990) and that it enhances the rate of neuromuscular reinnervation following partial denervation (Langfeld-Oster et al., 1994).

The tightly and differentially regulated pattern of NCAM isoform expression during in vivo neuromuscular development suggests that these alternately spliced isoforms are playing distinct roles. Since only the 145-kD isoform is present when primary myotubes form and when nerves first branch and synapse within muscle, it is most likely responsible for mediating some of the NCAM-dependent nerve–muscle interactions described above. The up-regulation of the 130-kD isoform only at the onset of secondary myogenesis and its persistence in the adult suggests that it may be playing other roles. However, thus far there is only limited in vitro evidence on different effects of NCAM isoforms. Specifically, Peck and Walsh (1993) have shown that the 140-kD isoform is more effective in enhancing myotube fusion in culture than the GPI-linked MSD containing isoform. Clearly, further experiments that specifically addresss this question, both in vitro and in the animal are needed.

In summary, the culture paradigm that we have developed should be a useful one in which to further elucidate the cellular pathways by which overall NCAM gene transcription as well as the synthesis of alternatively spliced

isoforms is regulated. In addition, the ability to switch in culture the predominant NCAM isoform expressed by these myotubes, should allow us to better determine the role played by individual isoforms in muscle–muscle cell interactions such as myoblast alignment, fusion, and separation, as well as in nerve–muscle interactions, including neurite growth, branching, and the formation and stabilization of synapses.

We wish to thank Dr. Urs Rutishauser for supplying the NCAM antibodies and to Roman Fatkhiyev for his technical assistance. For critical reading of the manuscript, we are grateful to Louise Milner, Mary-Anne Usiak, Drs. Esther Stoeckli, and Urs Rutishauser.

This work was supported by a National Science Foundation grant IBN 94 96177, and by National Institutes of Health grants, NS19640 from National Institute of Neurological Disorders and Stroke, HD 18369, and EY 06107. V.F. Rafuse was supported as a postdoctoral fellow funded by the Rick Hansen Man in Motion Legacy Foundation.

Received for publication 16 June 1995 and in revised form 27 December 1995.

References

Acheson, A., J.L. Sunshine, and U. Rutishauser. 1991. NCAM polysialic acid can regulate both cell-cell and cell-substrate interactions. *J. Cell Biol.* 114: 143–154.

Arevalo, J.I., and H.J. Saz. 1992. Effects of cholinergic agents on the metabolism of choline in muscle from *Ascaris Suum*. *J. Parasitol.* 78:387–392.

Asaoka, Y., S.-I. Nakamura, K. Yoshida, and Y. Nishizuka. 1992. Protein kinase C, calcium and phospholipid degradation. *Trends Biochem. Soc.* 17: 414–417.

Bartsch, U., F. Kirchhoff, and M. Schachner. 1990. Highly sialylated NCAM is expressed in adult mouse optic nerve and retina. *J. Neurocytol.* 19:550–565.

Bekoff, A., P.S.G. Stein, and V. Hamburger. 1975. Coordinated motor output in the hindlimb of the 7-day chick embryo. *Proc. Natl. Acad. Sci. USA.* 72: 1245–1248.

Berg, D.K., R.B. Kelly, P.B. Sargent, P. Williamson, and Z.W. Hall. 1972. Binding of alpha-bungarotoxin to acetylcholine receptors in mammalian muscle. *Proc. Natl. Acad. Sci. USA.* 69:147–151.

Betz, H., and J.-P. Changeux. 1979. Regulation of muscle acetycholine receptor synthesis in vitro by derivatives of cyclic nucleotides *in vitro*. *Nature (Lond.).* 278:749–752.

Booth, C.M., S.K. Kemplay, and M.C. Brown. 1990. An antibody to neural cell adhesion molecule impairs motor nerve terminal sprouting in a mouse muscle locally paralyzed with botulinum toxin. *Neuroscience.* 35:85–91.

Breen, K.C., and C.M. Regan. 1988. Differentiation-dependent sialylation of individual neural cell adhesion molecule polypeptides during postnatal development. *J. Neurochem.* 50:712–716.

Breen, K.C., P.G. Kelly, and C.M. Regan. 1987. Postnatal D2-CAM/NCAM sialylation state is controlled by a developmentally regulated Golgi sialyltransferase. *J. Neurochem.* 48:1486–1493.

Bruses, J.L., S. Oka, and U. Rutishauser. 1995. NCAM associated polysialic acid on ciliary ganglion neurons is regulated by polysialyltransferase levels and interaction with muscle. *J. Neurosci.* 15:8310–8319.

Byeon, M.K., Y. Sugi, R.R. Markwald, and S. Hoffman. 1995. NCAM polypeptides in heart development: Association with Z Discs of forms that contain the muscle-specific domain. *J. Cell Biol.* 128:209–221.

Cerny, L.S., and E. Bandman. 1986. Contractile activity is required for the expression of neonatal myosin heavy chains in embryonic chick pectoral muscle cultures. *J. Cell Biol.* 103:2153–2161.

Changeux, J.-P., C. Babinet, J.L. Bessereau, A. Bessis, A. Cartaud, J. Cartaud, P. Daubas, A. Devellers-Thiery, A. Duclert, J.A. Hill, et al. 1990. Compartmentalization of acetylcholine receptor gene expression during development of the neuromuscular junction. *Cold Spring Harbor Symp. Quant. Biol.* 55: 381–396.

Corriveau, R.A., S.J. Romane, W.G. Conroy, L. Oliva, and D.K. Berg. 1995. Expression of neuronal acetylcholine receptor genes in vertebrate skeletal muscle development. *J. Neurosci.* 15:1372–1383.

Covault, J., and J.R. Sanes. 1985. Neural cell adhesion molecule (NCAM) accumulates in denervated and paralyzed skeletal muscle. *Proc. Natl. Acad. Sci. USA.* 82:4544–4548.

Covault, J., and J.R. Sanes. 1986. Disribution of NCAM in synaptic and extrasynaptic portions of developing and adult skeletal muscle. *J. Cell Biol.* 102: 716–730.

Covault, J., Q-y. Liu, and S. El-Deeb. 1991. Calcium-activated proteolysis of intracellular domains in the cell adhesion molecules NCAM and N-Cadherin. *Mol. Brain Res.* 11:11–16.

Covault, J., J.P. Merlie, C. Goridis, and J.R. Sanes. 1986. Molecular forms of N-CAM and its RNA in developing and denervated skeletal muscle. *J. Cell Biol.* 102:731–739.

Cremer, H., R. Lange, A. Christoph, M. Plomann, G. Vopper, J. Roes, R. Brown, S. Baldwin, P. Kraemer, S. Scheff, D. Barthels, K. Rajewsky, and W. Wille. 1994. Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. *Nature (Lond.).* 367: 455–459.

Cunningham, B.A., J.J. Hemperly, B.A. Murray, E.A. Prediger, R. Brackenbury, and G.M. Edelman. 1987. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science (Wash. DC).* 236:799–806.

Dickson, G., D. Peck, S.E. Moore, C.H. Barton, and F.S. Frank. 1990. Enhanced myogenesis in NCAM-transfected mouse myoblasts. *Nature (Lond.).* 344:348–351.

Dodd, J., S.B. Morton, D. Karasogeris, M. Yamaoto, and T.M. Jessel. 1988. Spatial regulation of axonal glycoprotein expression in subsets of embryonic spinal neurons. *Neuron.* 1:105–116.

Doherty, P., and F.S. Walsh. 1992. Cell adhesion molecules, second messengers and axonal growth. *Curr. Opin. Neurobiol.* 2:595–601.

Fontaine, B., A. Klarsfeld, and J.-P. Changeux. 1987. Calcitonin-gene related peptide and muscle activity regulate acetylcholine receptor alpha-subunit mRNA levels by distinct intracellular pathways. *J. Cell Biol.* 105:1337–1342.

Fredette, B.J., and L. Landmesser. 1991. A reevaluation of the role of innervation in primary and secondary myogenesis in developing chick muscle. *Dev. Biol.* 143:19–35.

Fredette, B.J., U. Rutishauser, and L. Landmesser. 1993. Regulation and activity-dependence of N-Cadherin, NCAM isoforms, and polysialic acid on chick myotubes during development. *J. Cell Biol.* 123:1867–1888.

Frelinger, A.L., III, and U. Rutishauser. 1986. Topography of NCAM structural and functional determinants. II. Placement of monoclonal antibody epitopes. *J. Cell Biol.* 103:1729–1737.

Goldman, D., H.R. Brenner, and S. Heinemann. 1988. Acetylcholine receptor α, β, and δ subunit mRNA levels are regulated by muscle activity. *Neuron.* 1: 329–333.

Hahn, C., and J. Covault. 1992. Neural regulation of N-Cadherin gene expression in developing and adult skeletal muscle. *J. Neurosci.* 12:4677–4687.

Hamburger, V., and H.L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49–92.

Harris, A.J. 1981. Embryonic growth and innervation of rat skeletal muscles. I. Neural regulation of muscle fibre numbers. *Philos. Trans. R. Soc. Lond. Biol. Sci.* 293:257–277.

Hoffman, S., K.L. Crossin, E.A. Prediger, B.A. Cunningham, and G.M. Edelman. 1990. Expression and function of cell adhesion molecules during the early development of the heart. *Ann NY Acad. Sci.* 588:73–86.

Huang, C.-F, B.E. Flucher, M.M. Schmidt, S.K. Stroud, and J. Schmidt. 1994. Depolarization-transcription signals in skeletal muscle use calcium flux through L channels, but bypass the sarcoplasmic reticulum. *Neuron.* 13:167–177.

Huang, C.-F., C.M. Neville, and J. Schmidt. 1993. Control of myogenic factor genes by the membrane depolarization/protein kinase C cascade in chick skeletal muscle. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 319:21–25.

Huang, C.-F., J. Tong, and J. Schmidt. 1992. Protein kinase C couples membrane excitation to acetylcholine receptor gene inactivation in chick skeletal muscle. *Neuron.* 9:671–678.

Klarsfeld, A., R. Laufer, B. Fontaine, A. Devillers-Thiery, C. Dubreuil, and J.P. Changeux. 1989. Regulation of muscle AChR alpha subunit gene expression by electrical activity: Involvement of protein kinase C and Ca^{2+}. *Neuron.* 2: 1229–1236.

Knudsen, K.A. 1990. Cell adhesion molecules in myogenesis. *Curr. Opin. Cell Biol.* 2:902–906.

Knudsen, K.A., L. Smith, and S. McElwee. 1989. Involvement of cell surface phospatidylinositol-anchored glycoporteins in cell-cell adhesion of chick embryo myoblasts. *J. Cell Biol.* 109:1779–1786.

Knudsen, K.A., S.A. McElwee, and L. Myers. 1990. A role for the neural cell adhesion molecule, NCAM, in myoblast interaction during myogenesis. *Dev. Biol.* 138:159–168.

Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature (Lond.).* 227:680–685.

Landmesser, L., L. Dahm, K. Schultz, and U. Rutishauser. 1988. Distinct roles for adhesion molecules during innervation of embryonic chick muscle. *Dev. Biol.* 130:645–670.

Langfeld-Oster, B., A. Faissner, A. Irintchev, and A. Wernig. 1994. Polyclonal antibodies against NCAM and tenascin delay endplate reinnervation. *J. Neurocytol.* 23:591–604.

Lomo, T., and R.H. Westgaard. 1975. Further studies on the control of Ach sensitivity by muscle activity in the rat. *J. Physiol. (Lond.).* 252:603–626.

Lyles, J.M., W. Amin, E. Bock, and C.L. Weil. 1993. Regulation of NCAM by growth factors in serum free myotube cultures. *J. Neurosci. Res.* 34:273–286.

Lyons, G.E., R. Moore, O. Yahara, M.E. Buckingham, and F.S. Walsh. 1992. Expression of NCAM isoforms during skeletal myogenesis in the mouse embryo. *Dev. Dynamics.* 194:94–104.

McDonald, K.A., A.F. Horwitz, and K.A. Knudsen. 1995. Adhesion molecules and skeletal myogenesis. *Semin. Dev. Biol.* 6:105–116.

Mege, R.M., D. Goudou, M. Nicolet, L. Garcia, G. Geraud, and F. Reiger. 1992. N-Cadherin and NCAM in myoblast fusion: compared localization and

effect of blockade by peptides and antibodies. *J. Cell. Sci.* 103:897–906.
Mendelzon, D., J.-P. Changeux, and H.-O. Nghiem. 1994. Phosphorylation of myogenin in chick myotubes: regulation by electrical activity and by protein kinase C. Implications for acetylcholine receptor gene expression. *Biochemistry.* 33:2568–2575.
Miledi, R., and L.T. Potter. 1971. The acetylcholine receptors in muscle fibers. *Nature (Lond.).* 233:599–603.
Moore, S.E., and F.S. Walsh. 1986. Nerve dependent regulation of neural cell adhesion molecule expression in skeletal muscle. *Neuroscience.* 18:499–505.
Müller-Decker, K. 1989. Interrruption of TPA-induced signals by an antiviral and antitumoral xanthate compound: inhibition of a phospholipase C-type reaction. *Biochem. Biophys. Res. Commun.* 162:198–205.
O'Donovan, M.J., and L. Landmesser. 1987. The development of hindlimb motor activity studied in the isolated spinal cord of the chick embryo. *J. Neurosci.* 7:3256–3264.
Ono, K., H. Tomasiewicz, T. Magnuson, and U. Rutishauser. 1994. N-CAM mutation inhibits tangential neuronal migration and is phenocopied by enzymatic removal of polysialic acid. *Neuron.* 13:595–609.
Owens, G.C., G.M. Edelman, and B.A. Cunningham. 1987. Organization of the neural cell adhesion molecule (NCAM) gene: alternative axon usage as the basis for different membrane-associated domains. *Proc. Natl. Acad. Sci. USA.* 84:294–298.
Peck, D., and F.S. Walsh. 1993. Differential effects of overexpressed neural cell adhesion molecule isoforms on myoblast fusion. *J. Cell. Biol.* 123:1587–1595.
Pette, D., and G. Vrbova. 1992. Adaptation of mammalian skeletal fibers to chronic electrical stimulation. *Rev. Physiol. Biochem. Pharmacol.* 120:115–202.
Richter, E.A., P.J.-F. Cleland, S. Rattigan, and M.G. Clark. 1987. Contraction-associated translocation of protein kinase C in rat skeletal muscle. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 217:232–236.
Ross, J.J., M.J. Duxson, and A.J. Harris. 1987. Formation of primary and secondary myotubes in rat lumbrical muscles. *Development.* 100:383–394.
Shainberg, A., S.A. Cohen, and P.G. Nelson. 1976. Induction of acetylcholine receptors in muscle cultures. *Pflugers. Arch.* 361:255–261.
Sheppard, A., J. Wu., U. Rutishauser, and G. Lynch. 1991. Proteolytic modification of neural cell adhesion molecule (NCAM) by the intracellular proteinase calpain. *Biochim. Biophys. Acta.* 1076:156–160.
Stallcup, W.B. 1977. Comparative pharmacology of voltage-dependent sodium channels. *Brain Res.* 135:37–53.
Tamaoki, T., H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto, and F. Tomita. 1986. Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.* 135:397–402.
Tang, J., and L. Landmesser. 1993. Reduction in intramuscular nerve branching and synaptogenesis is correlated with decreased motoneuron survival. *J. Neurosci.* 13:3095–3103.
Tassin, A.M., R.M. Mege, D. Goudou, G.M. Edelman, and F. Rieger. 1991. Modulation of expression and cell surface distribution of N-CAM during myogenesis in vitro. *Neurochem. Int.* 18:97–106.
Thastrup, O., P.J. Cullen, B. Drøbak, M.R. Hanley, and A.P. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. USA.* 87:2466–2470.
Tosney, K.W., M. Watanabe, L. Landmesser, and U. Rutishauser. 1986. The distribution of NCAM in the chick hindlimb during axon outgrowth and synaptogenesis. *Dev. Biol.* 114:437–452.
Walsh, K.B., S.H. Bryant, and A. Schwartz. 1986. Effect of calcium antagonist drugs on calcium currents in mammalian skeletal muscle fibers. *J. Pharm. Exp. Therap.* 236:403–407.
Watanabe, M., A.L. Frelinger, and U. Rutishauser. 1986. Topography of N-CAM structural and functional determinants. I. Classification of monoclonal antibody epitopes. *J. Cell Biol.* 103:1721–1727.
Yoshimi, T., N. Mimura, S. Aimoto, and A. Asano. 1993. Transitional expression of neural cell adhesion molecule isoforms during chicken embryonic myogenesis. *Cell. Struct. Funct.* 18:1–11.